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RESEARCH

The Bacterial and Viral Communities Associated with Onion Bacterial Bulb Rot

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ABSTRACT

Microbes occur in complex communities within plants as endophytes and establish a network of interactions that can influence plant health positively or negatively. Positive interactions may be synergistic and confer abiotic and biotic stress tolerance. The aim of this study was to identify bacteria and viruses present in storage onion bulbs that were symptomatic and asymptomatic for bacterial bulb rot from crops grown in Georgia and Washington states, and to assess their potential role in the bulbs based on the functions of bacterial and viral genes detected. DNA was extracted from nine asymptomatic bulbs and nine bulbs displaying symptoms of bacterial bulb rot and subjected to 16S rRNA amplicon sequencing and metagenome-assembled genome analysis. The Illumina platform was used to sequence the hypervariable region (V3-V4) of the 16S rRNA gene. The 16S rRNA amplicon profiling revealed the presence of numerous bacteria, including potential onion pathogens in the genera *Pantoea* and *Burkholderia*. Metagenome-assembled genome assembly identified *P. agglomerans*, *B. gladioli*, and *B. cepacia*, known bulb rot pathogens, including genes linked to fitness and those involved in both type II and III secretion systems. Eighty-nine unique viral genomes were identified, of which 67 could be classified taxonomically. The bacterial and viral genomes differed significantly in asymptomatic versus symptomatic bulbs. Viral genomes showed evidence of auxiliary metabolic genes, including genes involved in fitness and pathogenicity to bacterial hosts. The onion bulbs hosted endophytic bacteria and viruses, some of which were potentially beneficial and others potentially pathogenic to onion or as hosts to bacteriophages.

Keywords: amplicon sequencing, metagenome assembled genomes (MAGs), onion bulb rot

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Onions (*Allium cepa* L.) are an economically important vegetable and one of the most valuable crops in the world. Current global production is estimated at 106.6 million tons, having increased by nearly 25% in the last 10 years. The largest producers are India (25.99%), China (22.67%), Egypt (3.11%), the United States (2.91%), and Turkey (2.55%) (FAO 2023). The United States produced almost 3.1 million tons of onion bulbs in 2022, with an economic value of $1.63 billion (Shahbandeh 2023). Approximately 68% of the annual crop produced is intended for storage (United States Department of Agriculture National Agricultural Statistics Service 2017). Bulb rot, caused by fungal or bacterial pathogens, is a substantial element of onion loss in storage, with bacterial pathogens alone causing around $16 million in damage annually in the United States (MacKay et al. 2022).

Internal tissues of plants commonly are inhabited by endophytic microbial communities that interact with the host plants as symbionts, commensals, or pathogens. Plants and their endophytes typically exist in a state of "balance," but if the relationship is disturbed

significantly by external or internal factors, the interaction may be disrupted, resulting in a shift from a mutualistic to a pathogenic lifestyle of the endophyte (Kogel et al. 2006; Ludwig-Müller 2015; Porter and Sachs 2020; Rudgers et al. 2020). These pathogens are regarded as opportunists. In the case of onion bulb rots, the high temperature typically associated with field-curing in warm regions or with postharvest curing is conducive to the growth and development of thermophilic bacteria that are pathogens of onion bulbs (Schroeder and du Toit 2010; Schroeder et al. 2012; Vahling-Armstrong et al. 2016).

The co-occurrence of multiple pathogens in a plant host may increase the severity of some plant diseases (Lamichhane and Venturi 2015). For example, several *Pantoea* spp. can coexist and co-express virulence in onion tissues (Vahling-Armstrong et al. 2016). Coinfection can also occur between distantly related species. For example, the primary pathogen causing olive knot, *Pseudomonas savastanoi* pv. *savastanoi*, causes larger knots when plants are co-colonized by the nonpathogenic species *Erwinia oleae*, *Erwinia toletana*, and *Pantoea agglomerans* (Buonaurio et al. 2015).

The primary bulb rot pathogens of onion are species of *Burkholderia*, *Enterobacter*, *Pantoea*, and *Pectobacterium* (Schwartz and Mohan 2008). However, some opportunistic bacterial pathogens have also been associated with rots on onion bulbs, including *Bacillus amyloliquefaciens* (Hwang et al. 2012) and species of *Dickeya* (Ma et al. 2020; Palacio-Bielsa et al. 2007), *Pseudomonas* (Gitaitis et al. 2012; Kim et al. 2002; Sawada et al. 2021), *Rahnella* (Asselin et al. 2019), and *Serratia* (Kowalska et al. 2015; Ovcharenko et al. 2011). Some of these species infect onion plants during the field season and may cause foliar symptoms, from which the infections progress down the neck into the developing bulb (Conn et al. 2012). Seeds harvested from symptomatic and asymptomatic plants in onion seed crops can harbor a few of these onion bacterial pathogens (Dutta and Gitaitis 2020; Humeau et al. 2006; Moloto et al. 2020).

Bacteriophages are ubiquitous in nature and have been shown to influence the structure, function, and evolution of microbial communities and to act as agents of lateral gene transfer (Sommers et al. 2021). In human microbiome studies, for example, the dominant members of commensal microbiota are bacteriophages (Minot et al. 2011; Pride et al. 2012; Reyes et al. 2010) that make up what is known as the phageome. Viral plant metagenomics focused on viral discovery have revealed a multitude of pathogenic and mutualistic relationships between viruses and plants (Roossinck et al. 2015). Pratama et al. (2020) proposed that, due to the similarity in complexity between the gut microbiome and the rhizosphere, phages potentially could drive the composition and functionality of the rhizosphere microbiome, including interactions with the plant host. In a virome study by Forero-Junco et al. (2022) on the wheat phyllosphere, the rhizosphere habitat was shown to harbor abundant and diverse bacteriophages, with the potential for these phages to act as biocontrol agents against phytopathogenic bacteria or as microbiome modulators that favored plant growth-promoting bacteria.

In this study, the diversity of bacterial and viral communities was analyzed in asymptomatic onion bulbs as well as in onion bulbs displaying symptoms of bacterial rot. The bulbs were grown in Georgia and Washington states in the United States and retrieved after they had been placed in storage facilities in each state. The objectives were to (i) determine if there were differences in bacterial and viral communities detected in the symptomatic versus asymptomatic bulbs, (ii) assess if the bacterial and viral communities differed between farms (states) in which the bulbs were grown and stored, and (iii) examine the genes detected in the bacteria and viruses in these bulbs that might contribute to survival as well as a pathogenicity to onion (for bacteria detected) or bacteria (for bacteriophages detected).

MATERIALS AND METHODS

Sampling strategy. Onion bulb samples of the cultivar Redwing (a common red storage cultivar grown in the Columbia Basin of Washington State in the United States) were collected in May 2020 from a commercial storage facility in Franklin County, Washington. The onions were grown in the semiarid production region of central Washington in 2019 using center pivot irrigation. The onion crop was sown in the spring of 2019 and harvested in September 2019 after undercutting (severing of the roots to facilitate field-curing) and mechanical topping typical for the Columbia Basin. After harvest, the bulbs were stored in a commercial storage facility under conditions typical for Columbia Basin storage cultivars (i.e., at approximately 4 to 5°C and 70% relative humidity).

Bulbs of the yellow cultivar Vidora were sampled from storage in May 2020 at the Tifton field site at the University of Georgia in the Vidalia region of sweet onion production in Georgia. The crop was grown from December 2019 to May 2020 using overhead risers for irrigation. There were several rainfall events over the season, particularly in February and April 2020. The temperature throughout the growing season ranged from 4 to 12°C in January to mid-March, and then exceeded 21°C during April and May. The crop was harvested using undercutting and hand-clipping practices typical for the Vidalia region and field-cured for 3 days prior to harvest. The University of Georgia Fungicide Program (https://site.extension.uga.edu/benhillcoag/files/2022/04/Fungicide-Spray-Programs-2022_vegetable-crops.pdf) was followed, apart from copper applications to allow for natural progression of bacterial diseases. After harvest, onion bulbs were kept in a storage facility (0°C and relative humidity of 65 to 70%) until shipment to markets.

DNA extraction and sequencing. Nine asymptomatic onion bulbs and nine bulbs from the same storage rot that had symptoms of bacterial rot were sampled from each state. The dry, outer scales were removed, and each bulb was cut vertically through the next to the basal plate with a sterilized knife to reveal the internal fleshy scales of the bulbs (Fig. 1). For the symptomatic onion bulbs, a small amount of fleshy scale tissue was excised, weighed (approximately 5 g), and homogenized in 1× phosphate-buffered solution using a universal extraction bag (Bioreba AG). The asymptomatic bulbs were processed similarly by excising the same amount of fleshy scale tissue from a similar location to the sample collected from symptomatic bulbs. DNA was extracted using a DNeasy PowerSoil kit (QIAGEN) according to the manufacturer's protocol. The extracted DNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and stored at −20°C.

Eluted DNA extracted samples of the 18 asymptomatic and 18 symptomatic onion bulbs were sent to Admera Health (South Plainfield, NJ, U.S.A.) for sequencing. Sequencing of the V3-V4 hypervariable region (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′; Klindworth et al. 2013) of the 16S rRNA gene was performed on an Illumina 2 × 250-bp platform with a sequencing depth of approximately 100,000 reads per sample (50,000 per direction).

For metagenomic sequencing, an equivalent amount of DNA from each of the nine bulbs was pooled into one sample equating to 1 ng of DNA, representing one asymptomatic pooled bulb sample and one pooled symptomatic bulb sample for each of Georgia and Washington states. The pooled DNA samples were sent to

Admera Health for library preparation and sequencing. Next-generation sequencing was performed on an Illumina PE150 Cycle platform with read lengths of 150-bp pairs and a sequencing depth of approximately 120 million reads per sample (60 million per direction).

Bioinformatic analysis: 16S rRNA amplicons. The sequenced reads were filtered and assembled with the QIIME2 (Bolyen et al. 2019) pipeline, using DADA2 (Callahan et al. 2016) for filtering the reads and for unique sequence interference. Quality-based truncation lengths of 215 to 250 bp were used to filter reverse and forward reads. The number of raw sequence reads before and after DADA2 processing can be found in Supplementary Table S1. These were clustered into unique representative sequences, also referred to as amplicon sequence variants (ASVs). The ASV table, showing absolute counts for each ASV per sample, was generated using the QIIME2 pipeline. ASVs subsequently were classified taxonomically using the SILVA132 classifier (Quast et al. 2013) for prokaryotic species with a 99% similarity cutoff. To assess if the sequencing depth for each sample was adequate, rarefaction curves were generated using the Vegan package (Oksanen et al. 2019) in RStudio. Logarithmic diversity plots were then created using the Python-based package, Seaborn (Bisong 2019). Co-occurrence of taxa was noted from the ASV table, and a co-occurrence diagram was drawn.

Alpha-diversity metrics, beta-diversity metrics, and ordination were calculated using the ASV data with the Phyloseq (McMurdie and Holmes 2013) and Vegan packages in RStudio. Inverse Simpson, Shannon, and observed alpha-diversity indices were calculated. The distribution of the relative abundances and alpha-diversity indices was tested using the Shapiro-Wilk normality test (Royston 1982), and the significance of differences in phylum relative abundances and alpha diversity was calculated using a Welch two-sample *t* test (for normally distributed data) or the Wilcoxon rank-sum test (for non-normally distributed data) (Haynes 2013). To perform beta-diversity analyses, the ASV count table was first rarefied, using the sample with the lowest ASV number as the reference sample, and the counts were then $\log(x + 1)$ transformed. Beta diversity between groups was calculated using the Bray-Curtis dissimilarity metric (Beals 1984) and visualized using a principal coordinates analysis plot (Jolliffe and Cadima 2016). Permutational multivariate analysis of variance (Anderson and Walsh 2013) with 999 permutations was calculated to test for statistical differences in beta diversity between samples, and the variation within sample groups was tested using the analysis of multivariate homogeneity of group dispersions (β-disper) (Anderson 2006).

Bioinformatic analysis: Metagenomes. The raw sequence data for the pooled asymptomatic and symptomatic onion bulb samples from each state were processed before assembly. To assess the quality of the reads, FastQC v0.11.9 (Andrews 2010) was used to determine the integrity of the sequence data generated. Trimmomatic v0.40 (Bolger et al. 2014) was then used to remove the adapter on the 5′ end of the sequence, as well as the low-quality segments located on the 3′ end of the sequences and to create paired forward (R1) and reverse (R2) reads, with additional files for the singletons (S1, S2) using default parameters. Following this step, the paired reads (R1, R2) were merged using the IDBA v1.1.3 (Peng et al. 2012) assembler. This assembler uses the de Bruijn graph algorithm to fragment reads into smaller fragments (kmers) that are then aligned to form longer stretches of sequences (contigs). As the IDBA assembler generates several assembled datasets due to multiple kmer size parameters (kmer range; 33, 53, 73) (Peng et al. 2012), the quality of each iteration was assessed using Quast v5.0.2 (Gurevich et al. 2013). Using the highest-quality iterations of the assembled datasets, Kraken2 v2.1.0 (Wood et al. 2019) was used to determine the taxonomy of the sequences. This was then visualized using Krona v2.8.1 (Ondov et al. 2011). All contigs with eukaryotic or unknown/unclassified annotations were subsequently removed from the assemblies. The open reading frames of the contigs were predicted using Prodigal v2.6.3 (Hyatt et al. 2012), which produced FASTA files of the predicted nucleotide and protein sequences. The open reading frames were classified functionally using DIAMOND v0.9.21 (Buchfink et al. 2015), which uses a BLAST-based tool against a reference database of prokaryotic genes.

Three methods were used for binning of the metagenome-assembled genomes (MAGs): CONCOCT v1.0.0 (Alneberg 2018), MaxBin v2.2.4 (Wu et al. 2014), and MetaBAT v0.32.4 (Kang et al. 2015). To assess the completeness and contamination of the MAGs, CheckM (Parks et al. 2015) was used. After filtering the MAGs for

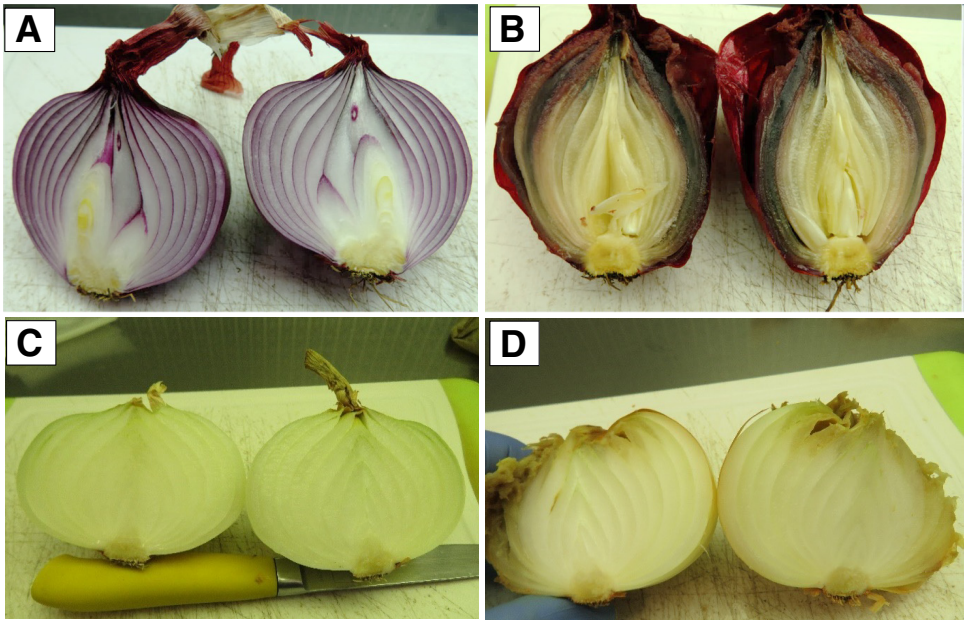

Fig. 1. Examples of onion bulbs of the cultivar Redwing sampled from storage in Washington **(A,** asymptomatic; **B,** symptomatic) and the sweet onion cultivar Vidora sampled after harvest in Georgia (**C,** asymptomatic; **D,** symptomatic). Each bulb was cut lengthwise to sample internal fleshy scales for DNA extraction.

a minimum of 50% completeness and a maximum of 10% contamination, the contigs of each binned genome were combined into longer scaffolds using MEDUSA (Karlsson et al. 2014).

Genes in the MAGs were predicted and annotated using the Rapid Annotations using Subsystems Technology toolkit (Meyer et al. 2008). This allowed for preliminary taxonomic analysis by using NCBI BLAST (Boratyn et al. 2013) analysis on the following predicted multilocus sequence typing genes: carbamate kinase, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triosephosphate isomerase, and acetyl-CoA acetyltransferase. The predicted taxonomy was confirmed by comparing the MAGs with reference genomes using OrthoANI (Lee et al. 2016). BBMap (Bushnell 2014) was then used to map the MAGs against the whole-metagenome datasets to determine the gene abundances within the metagenomes.

The genomes were analyzed to identify important genetic elements thought to aid in pathogenicity and survival in the onion environment. Using CLC Main Workbench 20.0.2 (QIAGEN), several genes of interest were identified from the Rapid Annotations using Subsystems Technology toolkit with regard to plant detoxification, virulence, and secretion systems and used in a BLAST-based search. The presence or absence of the genes was recorded and visualized using Seaborn (Bisong 2019).

Viral genome assembly. Viral contigs were identified from the assembled metagenome using VirSorter (Roux et al. 2015) on the iVirus platform hosted by Cyverse (Bolduc et al. 2017), using the virome database and the microbial decontamination option. Only predictions of categories 1, 2, 4, and 5 were used (phages and prophages identified with the "pretty sure" and "quite sure" qualification). The completeness and quality of extracted viral contigs was tested further using CheckV (Nayfach et al. 2021). The software VConTACT2 (Jang et al. 2019) was used for taxonomic assignments of the extracted viral contigs, which was confirmed using the Viral/Spacer blast function on the IMG/VR website (https://img.jgi.doe.gov/cgi-bin/vr/main.cgi). Predicted bacterial hosts for each viral contig were identified by screening each taxonomic annotation from the VConTACT2 results against the Virus-Host database (https://www.genome.jp/virushostdb/). These results were displayed as a network in Cytoscape V3.7 (Shannon et al. 2003), using the correlation score of the taxonomy assignments from the VConTACT2 results as the strength of the interaction values for the connections (edges) between viral and host nodes. In addition, functional annotations of predicted viral contigs, including the presence of auxiliary metabolic genes (AMGs), were mapped using the tool DRAMv1 (Shaffer et al. 2020).

RESULTS AND DISCUSSION

Community composition of endophytes in asymptomatic and symptomatic onion bulbs. The sequencing of the V3-V4 16S rRNA hypervariable region resulted in large chloroplast and cyanobacterial background signals (>90% of ASVs; Supplementary Fig. S1A). The cyanobacterial signal was presumed also to represent chloroplast reads. Both were removed prior to the biodiversity comparison between samples from asymptomatic and symptomatic onion bulbs from each state. The percentage of bacterial (not chloroplast or cyanobacterial) ASVs in the symptomatic bulbs was significantly greater (*t* test, $P < 0.05$) than in the asymptomatic samples, which is consistent with the expected bacterial communities in symptomatic bulbs (Supplementary Fig. S1B).

Taxonomic composition analysis of the bacterial dataset revealed distinct bacterial communities in the symptomatic and asymptomatic bulbs at the two sampling locations (Fig. 2). After removal of the host ASV signatures, bacterial ASVs could not be detected in eight of the nine asymptomatic bulb samples from Washington (Fig. 2A), suggesting that these bulbs did not contain detectable bacterial communities (i.e., the bacterial DNA dropped below the level of detection). The high level of onion signal contamination may have resulted in insufficient sequencing depth to capture the full extent of endophytic bacterial communities in these asymptomatic bulbs. Symptomatic bulbs from the Washington site had highly variable bacterial communities, with members from the orders *Enterobacterales*, *Burkholderiales*, and *Acetobacterales* being the most ubiquitous across the samples. This was expected, as members of the first two orders contain species that are onion bulb rot pathogens (Schwartz and Mohan 2008). This would include species in the *Enterobacterales*, such as *Pantoea agglomerans* (Edens et al. 2006), *P. ananatis* (Gitaitis and Gay 1997), *P. stewartii* subsp. *indologenes* (Stumpf et al. 2018), *Enterobacter ludwigii* (Schroeder et al. 2010), *Pectobacterium carotovorum* (Ma et al. 2007), and *Dickeya fangzhongdai* (Ma et al. 2020), and in the *Burkholderiales*, *Burkholderia cepacia* and *B. gladioli* pv. *allicola* (Gitaitis and Nischwitz 2007). By comparison, asymptomatic bulbs from Georgia were dominated by the bacterial orders *Enterobacterales* and *Pseudomonadales*, suggesting that bacterial communities in these bulbs harbored bacterial taxa that could be pathogenic to onion, although many species in these orders are not pathogens of onion (Fig. 2B). Pathogenic species in the *Pseudomonadales* capable of causing bulb rot include, for example, *Pseudomonas viridiflava* (Tsuji and Fuji 2021), *P. syringae* (Moloto et al. 2017), and *P. coronafaciens* (Dutta et al. 2018). There was a high relative abundance of the family *Pectobacteriaceae* in the asymptomatic bulbs from Georgia, which includes several taxa associated with soft rot of various vegetables (Fujimoto et al. 2021; B. Ma et al. 2007; X. Ma et al. 2020) (Supplementary Fig. S2) (i.e., *P. carotovorum* and *Dickeya* spp.). The bacterial communities of symptomatic onions stored in Georgia were less diverse than those from Washington State and were dominated by *Burkholderiales* and *Acetobacterales*, suggesting that bacterial infections of the onion bulbs from the two states were associated with distinct bacterial cohorts. In Washington State, the dominant bacterial communities were from the *Enterobacteriales* and the *Acetobacterales*. These results would suggest that the core pathobiome in symptomatic bulbs are species in the *Burkholderiales* in Georgia and the *Enterobacteriales* in Washington State. The abundance of *Acetobacterales* in some symptomatic bulbs from both locations could also be indicative of different temporal progression of bacterial infections, as these bacteria often associated with late-stage fermentation of infected plant tissue (Li et al. 2015). Similar results were reported by Yurgel et al. (2018), with 15 to 97% of DNA reads from some symptomatic onion bulbs annotated as members of the *Acetobacterales*. Most bacterial ASVs could only be annotated to family, highlighting the inadequacy of using the V3-V4 hypervariable region of the 16S rRNA gene for differentiation of closely related species in the *Enterobacterales* and *Burkholderiales* (Chakravorty et al. 2007).

The taxonomic composition of bacterial communities detected in onion bulbs grown in Georgia and Washington suggested that development of bacterial rot was associated with different communities in the bulbs. This was further shown by a significant difference ($P < 0.05$) in the Shannon alpha-biodiversity index between asymptomatic and symptomatic bulbs from both Washington (Fig. 3A) and Georgia (Fig. 3B). In both cases, the alpha diversity was significantly higher for the symptomatic bulbs, indicating a greater number and abundance of bacterial species in bulbs with bacterial rot. Although plant diseases are often linked to dysbiosis of the resident microbial communities, characterized by the loss of microbial diversity (Arnault et al. 2023), the significantly greater diversity observed

in this study for symptomatic bulbs correlated well with the greater count of bacterial reads in these bulbs compared with asymptomatic bulbs. Onions exhibit greater susceptibility to bulb infection close to harvest, particularly when their necks have reached maximum size, as they are capable of retaining larger amounts of water from rain or overhead irrigation (Belo et al. 2023). Once the onion tops fall over, a natural process of onion maturation, bacteria in the neck tissue reproduce on the senescing tissue, as there is still moisture in the necks, with infection moving down into the bulb (Brewster 2008). The combination of senescing tissue and moisture in the necks is highly favorable for the growth and colonization of pathogenic and opportunistic bacteria. A significant difference in community structure ($P < 0.05$) was also observed in bacterial communities detected in asymptomatic and symptomatic bulbs from both Georgia and Washington, with the symptomatic state (asymptomatic versus symptomatic) explaining 37% ($R^2 = 0.37$) of the observed community difference in the bulbs from Washington state (Fig. 3C) and 19% ($R^2 = 0.19$) of the difference in bulbs from Georgia (Fig. 3D). Host ASVs were included in the analysis to perform composition structure analysis, as the asymptomatic bulb data from Washington state did not contain enough bacterial community data for statistical analysis. As discussed above, asymptomatic onion bulbs are expected to have a diverse bacterial community based on research by Yurgel et al. (2018). Therefore, the lack of bacterial ASVs in 9 of the 10 asymptomatic Washington State bulbs was likely a reflection of low sequencing coverage, not the absence of bacteria present endophytically.

Functional traits associated with pathogenicity of bacterial communities in symptomatic onion bulbs. To access functional genetic markers of pathogenicity in the symptomatic onion bulbs, four metagenomes were sequenced, one for each of the asymptomatic and symptomatic onion bulbs from Georgia and likewise for Washington (one high-coverage metagenome per infection and geographic location). The metagenomes were assembled to generate contigs with gene coding information. Predicted taxonomic assignments of these contigs using Kraken2 yielded similar results to the amplicon sequencing results, with only 1% of the contigs in the asymptomatic bulb metagenomes and 2 and 5% of contigs

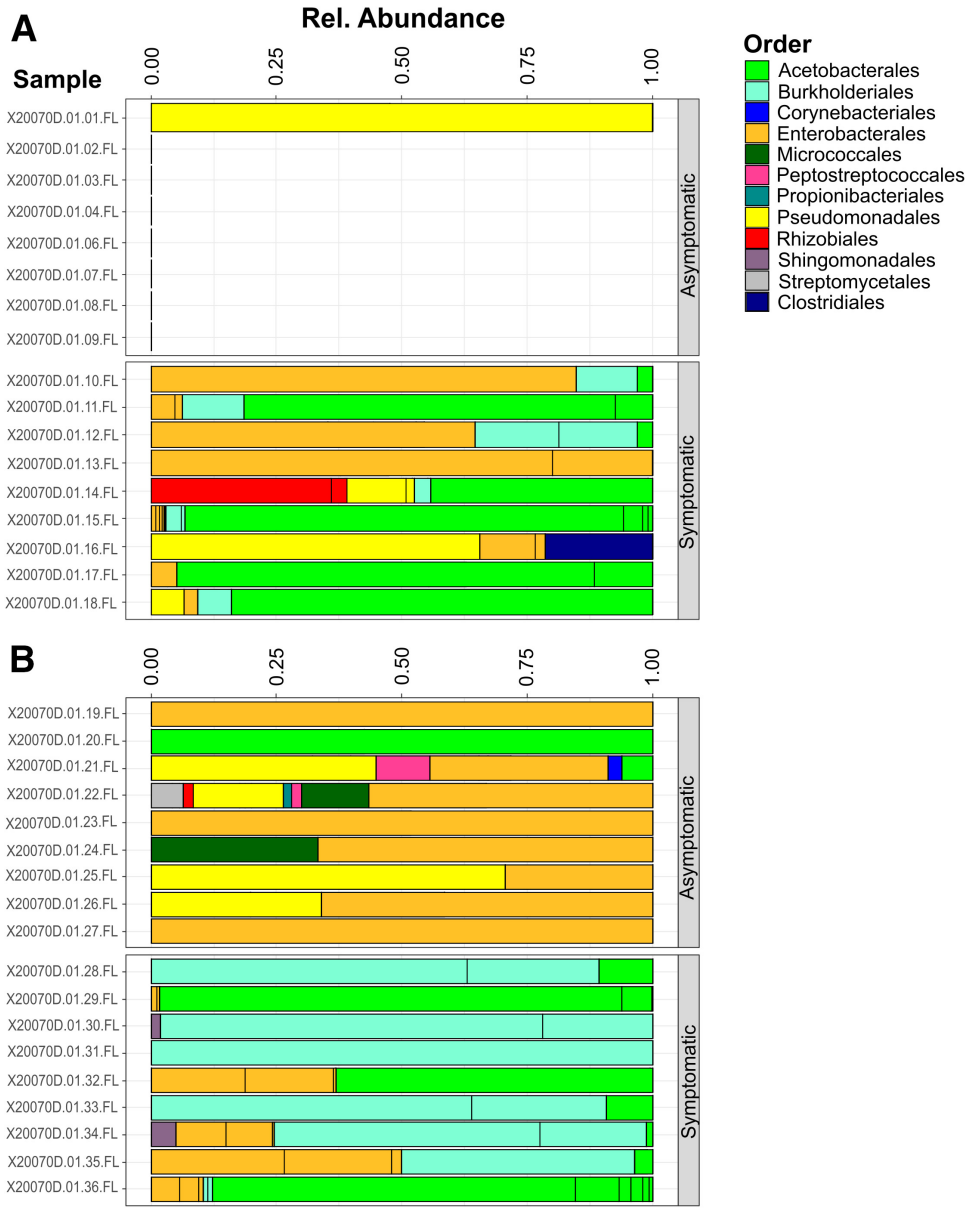

Fig. 2. Relative abundance of DNA of bacterial orders detected in asymptomatic onion bulbs and bulbs with symptoms of bacterial rot that were grown in **A,** Washington and **B,** Georgia, United States. Relative abundance was expressed as a fraction of total amplicon sequence variant (ASV) reads after removal of chloroplast and cyanobacterial ASVs from the plant host.

in the symptomatic metagenomes from Georgia and Washington samples, respectively, being annotated as bacterial contigs. However, contrary to the amplicon results, the pairs of asymptomatic and symptomatic metagenomes showed similar bacterial compositions at the taxonomic level of order (Fig. 4A). Specifically, bacterial communities of asymptomatic bulbs consisted of a diverse range of bacterial orders dominated by *Bacillales* and *Flavobacteriales*. By comparison, communities in symptomatic bulbs exhibited dysbiotic characteristics, with a high abundance of *Enterobacterales* and *Burkholderiales* and a lower abundance of bacterial orders *Bacillales* and *Flavobacteriales* associated with asymptomatic bulb microbiomes. Bacterial orders such as *Rhizobiales* and *Lactobacillales* include genera and species that often are associated with healthy plant microbiomes (Garrido-Oter et al. 2018; Jaffar et al. 2023) and that potentially can be used as biocontrol agents (Daranas et al. 2019). Their less abundant presence in the symptomatic bulbs suggests that these beneficial taxa may be outcompeted in bulbs during bacterial rot development. Focusing on the top five most abundant bacterial genera annotated from the contigs of the symptomatic bulb metagenomes revealed that, whereas the Georgia symptomatic samples were associated mostly with *Rahnella* and *Burkholderia* species, the Washington symptomatic bulb

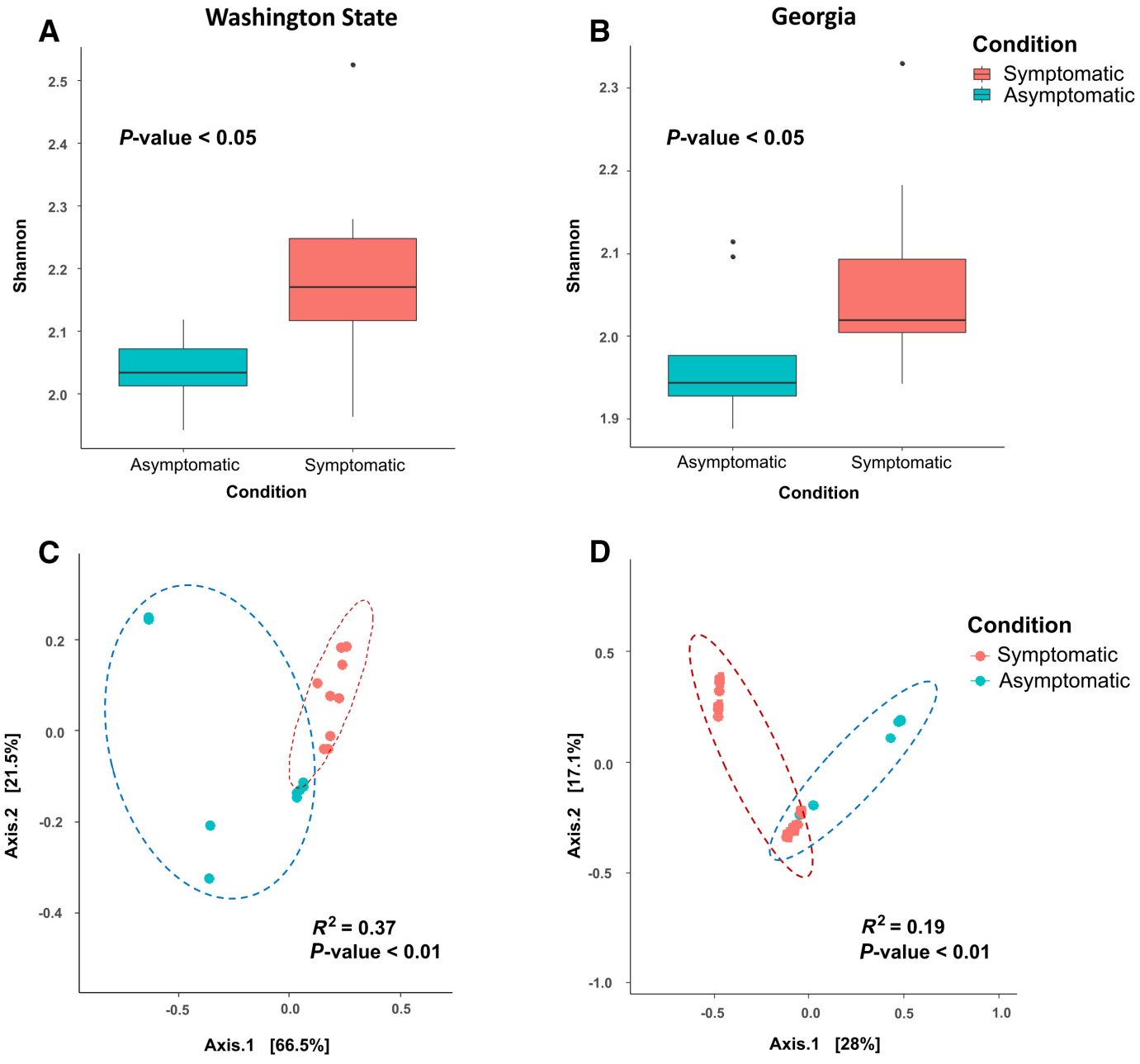

Fig. 3. Shannon alpha-diversity index for asymptomatic and symptomatic bulbs (bacterial bulb rot), as well as the principal coordinates analysis plots of the Bray-Curtis dissimilarity index between these bulbs grown in **A and C,** Washington and **B and D,** Georgia. The significance of the differences in alpha diversity between diseased state (asymptomatic vs. symptomatic) was calculated using the Wilcoxon rank-sum test. The significance of the beta-diversity differences between symptomatic bulbs was calculated using permutational multivariate analysis of variance. The *P* values and R^2 scores for the beta-diversity differences are shown in the respective plots.

microbiome also exhibited high proportions of genetic signals of *Pantoea vagans* and *Serratia liquefaciens* (Fig. 4B). Strains of all dominant species identified in the metagenomes of symptomatic bulbs, with the exception of *S. liquefaciens*, have been identified previously as onion pathogens that either cause severe disease symptoms (e.g., many *Burkholderia* and *Pantoea* strains) (Schwartz and Mohan 2008) or that are associated with opportunistic pathogenesis (e.g., *Rahnella* spp.) during onion infection (Asselin et al. 2019). These results suggest, similarly to the amplicon sequencing results, that progression of bacterial rot in onion plants or bulbs can be caused by distinct cohorts of pathogenic species coinfecting onion plants or bulbs, depending on the crop, cultivar, geographic region, and more.

To explore further the functional capacity of bacterial communities in onion bulbs, in particular functional traits associated with pathogenicity, the metagenomics data were resolved into MAGs. A total of six MAGs (five medium and one high quality, based on CheckM classifications) (Parks et al. 2015) were assembled from the metagenomics data, five of which were identified to species level (Supplementary Table S2). The taxonomy of the MAGs was consistent with the dominant families identified from taxonomic assignments of the metagenomics reads, with two MAGs belonging to *Burkholderia* species, one corresponding to *Pantoea agglomerans*, one to *Pseudomonas simiae*, one to *Acetobacter papayae*, and one to an unidentified *Rahnella* species. Except for *A. papayae*, all the other MAGs corresponded to taxa that have been identified previously as pathogens of onion. Based on normalized abundances (expressed as reads per kilobase per million), only two MAGs, those of *P. simiae* and the *Rahnella* sp., were detected in the asymptomatic bulb metagenome, whereas all MAGs were found in the symptomatic bulb data (Fig. 5A). This was not unexpected, considering that the metagenomic data from asymptomatic bulbs had very low bacterial read abundances, and most of the MAGs were associated with the symptomatic bulbs. The *Burkholderia* MAGs and *A. papayae* MAG were almost exclusively found in the metagenome of symptomatic onions from Georgia, whereas the *P. agglomerans*, *P. simiae*, and *Rahnella* sp. were mapped primarily to the metagenome of symptomatic onions from Washington. *Pseudomonas simiae* is a beneficial rhizobacterium (Pieterse et al. 2021), but its role, if any, as a plant pathogen is unknown. These results were consistent with those of both the taxonomic data from the metagenomes and the amplicons reads, which showed that bacterial communities in symptomatic onion bulbs from Georgia were dominated by *Burkholde-*

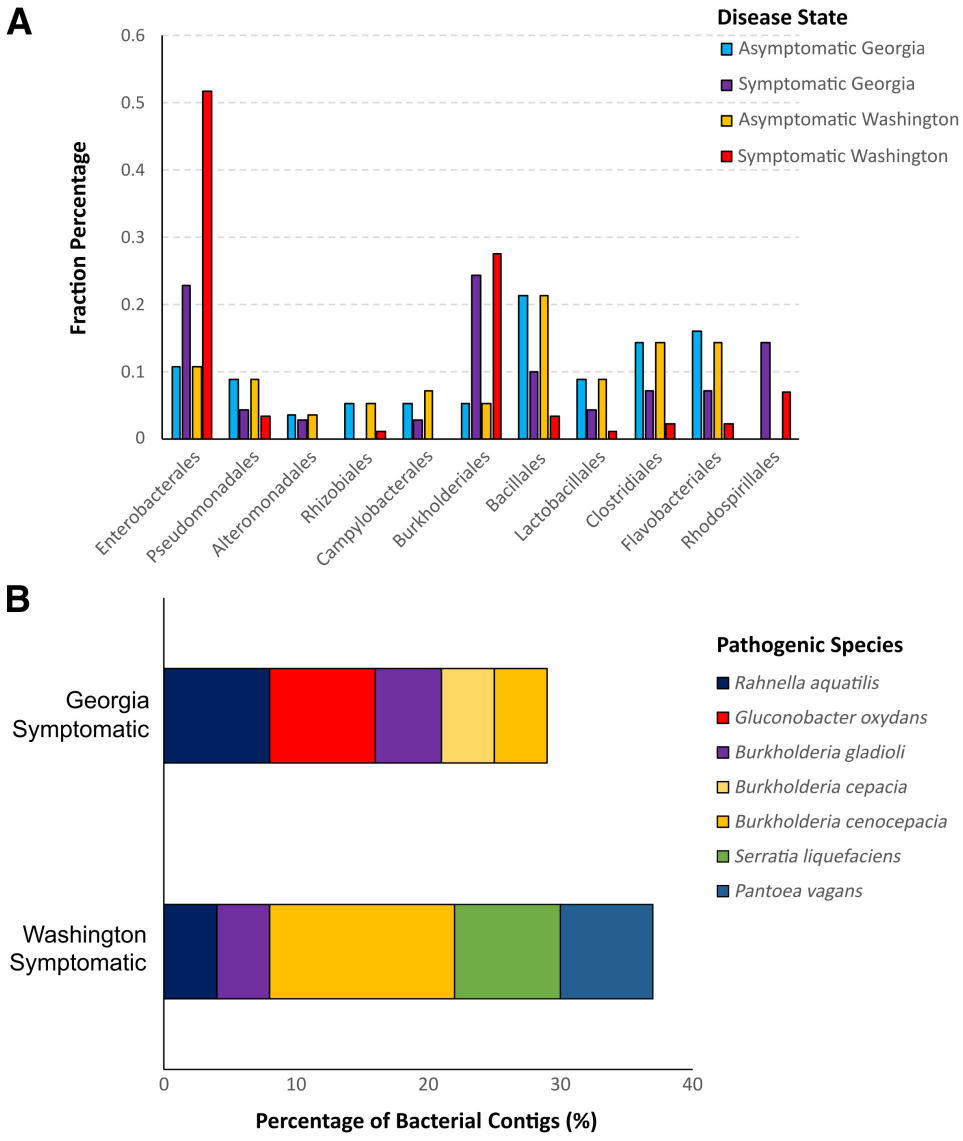

Fig. 4. A, Relative abundance (expressed as a fraction of total bacterial reads) of orders of bacteria with a relative abundance of ≥ 2% of the total reads across the metagenomes for bacterial communities from asymptomatic and symptomatic onion bulbs grown in Georgia and Washington. **B,** Relative abundance of identified pathogenic species that represented ≥ 2% of the total bacterial reads of the metagenomic data from symptomatic onion bulbs in Georgia and Washington.

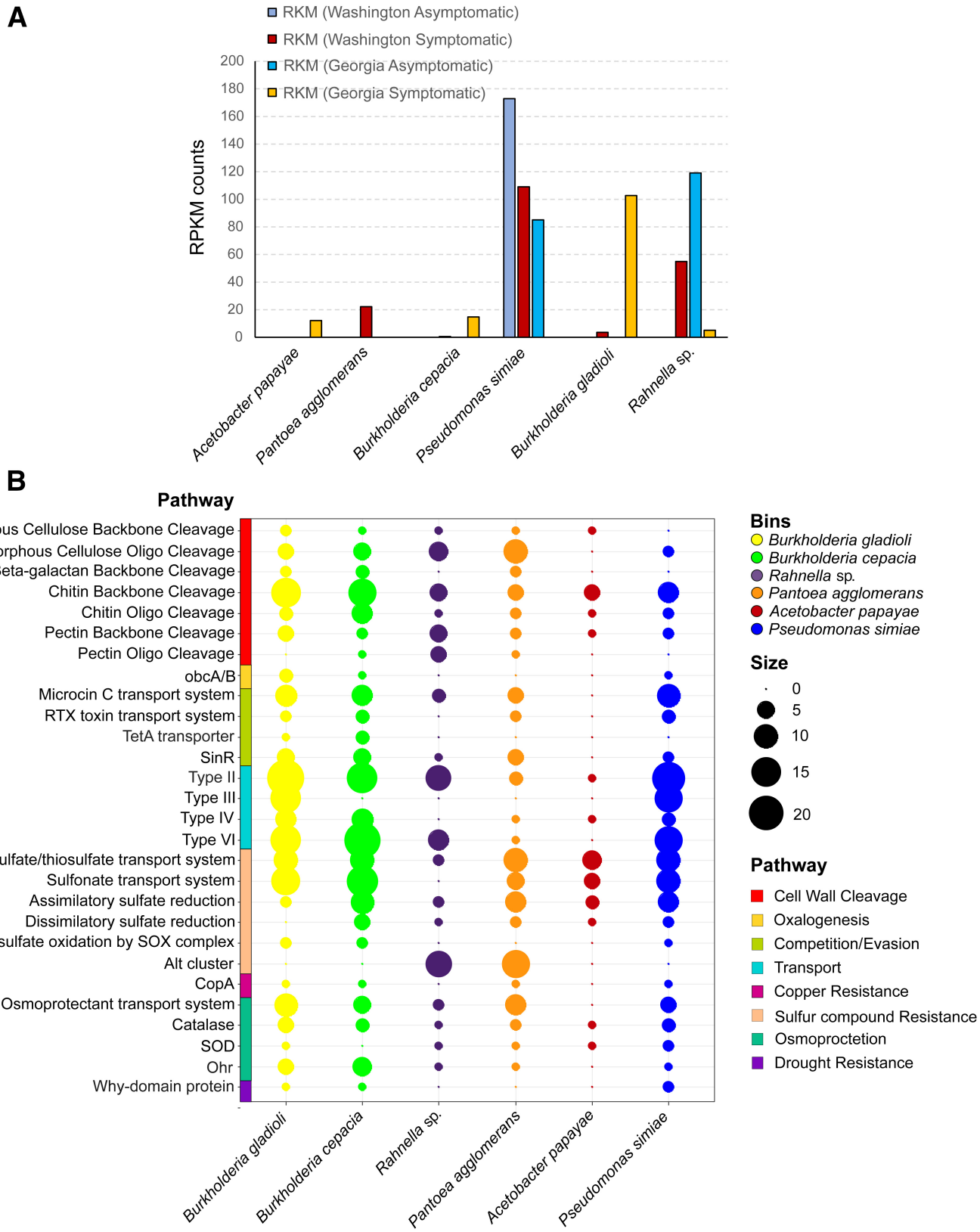

Fig. 5. **A,** Abundances of metagenome-assembled genomes (MAGs) for four metagenomic sequence data from asymptomatic vs. symptomatic onion bulbs in Georgia and Washington states. Abundance is expressed as reads per kilobase per million (RPKM). **B,** Bubble plot showing number of counts (size) for marker genes and biochemical processes potentially involved in pathogenicity to onion across the six MAGs. The presence and absence of genes for the pathways represented in the figure were based on the pfam/KEGG/CASy classifications obtained from the annotation pipeline. Individual marker genes were identified also and accounted for by screening the MAGs against custom DIAMOND databases. The size of the bubbles corresponds to the number of hits for a particular gene or pathway.

ria species, whereas taxa from the order *Enterobacterales* (such as *Pantoea* spp.) were found predominantly in bacterial communities in symptomatic onion bulbs from Washington State.

Screening the MAGs for pathogenicity-associated marker genes revealed that all the MAGs had genes associated with the ability to survive and grow in the fleshy scales of onion bulbs (Fig. 5B). For instance, all MAGs contained genes involved in the degradation of pectin and cellulose, with the *Burkholderia*, *Rahnella*, and *Pantoea* MAGs containing multiple genes that target these two plant cell-wall components. The *Burkholderia gladioli* MAG also contained a complete operon for oxalic acid biosynthesis, made of the oxalate biosynthetic component (obc)1 (*obcA*) and a gamma carbonic anhydrase family protein (*obcB*) (Nakata 2011), which has been associated with pathogenicity of both fungi and bacteria (Nakata and He 2010; Wang and Wang 2020). Both *B. gladioli* and *B. cepacia* MAGs exhibited a greater number of chitin degradation genes than the other assembled genomes, which is expected considering the role that members of *Burkholderia* have been shown to play in fungal biocontrol (Elshafie et al. 2012). In addition, all MAGs exhibited several genes involved in the transport and metabolism of organosulfur compounds, which could aid in their tolerance to antimicrobial compounds in fleshy onion scales, such as thiosulfinates (Stice et al. 2020). However, there is no evidence that any of these genes, other than those involved in thiosulfinate tolerance, contribute to this activity. Presence of the alt cluster, which plays a pivotal role in tolerance of *Pantoea* species to thiosulfinates present in onion tissue (Stice et al. 2021), was also observed in the *Rahnella* sp. MAG. To our knowledge, no previous studies have shown the presence of the alt cluster in *Rahnella* species, which suggests that *Rahnella* and *Pantoea* species may employ similar strategies to tolerate the chemical defense mechanisms in onion bulbs.

The assembled genomes showed several marker genes that impart tolerance to storage conditions of onion bulbs (Fig. 5B). MAGs for the *Burkholderia* and *Pseudomonas* species contained genes encoding Why domain-containing proteins. These are part of the group 2 LEA (late embryogenesis abundant) proteins, which are involved in the response to extreme dehydration and oxidative stress across all kingdoms of life (Mertens et al. 2018). Thus, the presence of these genes, in addition to the occurrence of other osmotic-resistance genes such as the *ohr* (organic hydroperoxide resistance) gene and an osmoprotectant transporter, suggest the *Burkholderia* and *Pseudomonas* genomes identified in the metagenomics data are well adapted to the dry conditions in which onions are grown in some regions (e.g., the semiarid Columbia Basin of Washington State) (Sánchez et al. 2020). In addition, copper-containing bactericides and fungicides are used commonly in onion production as preventive measures against plant pathogens (Lamichhane et al. 2018). Screening of the MAGs for copper resistance genes revealed that the *Burkholderia* and *Pantoea* genomes contained a gene encoding the copper resistance protein A (CopA), which is directly involved in resistance to copper (Bondarczuk and Piotrowska-Seget 2013). This suggests that these bacterial strains may be resistant to copper-containing bactericides used in onion production.

Overall, these results demonstrate that the MAGs from well-characterized plant pathogens extracted from the metagenomic data of symptomatic onion bulbs in Georgia and Washington, in particular the *Burkholderia* species, have the genetic arsenal that facilitates infection and persistence in onion bulb tissue, not only due to the genes discussed above, but also due to the presence of other genes commonly involved in pathogenicity (e.g., genes for the type II, III, IV, and VI transport systems) (Green and Mecsas 2016), as well as the gene for the biofilm and enterotoxin secretion regulator, SinR (Colledge et al. 2011). By comparison, the *A. papayae* MAG exhibited a low number or absence of these genes, confirming the 16S rRNA amplicon results that this species might be a secondary invader after initiation of infection on onion bulbs by primary bacterial pathogens.

Viral communities in asymptomatic and symptomatic onion bulbs. Viruses such as bacteriophages are well-characterized key players in the ecology of ecosystems by controlling population dynamics and directing nutrient cycles through the predation of their microbial hosts (Gazitúa et al. 2021). Bacteriophages have also been identified as promising biocontrol agents against several foodborne human pathogens (e.g., Imran et al. 2023) and plant pathogens (e.g., Jones et al. 2021). Thus, to investigate whether viral populations might play a role in onion bulb bacterial communities, the metagenomic data were screened for viral contigs using a combination of bioinformatics tools that detect metagenomic contigs containing viral genes. Based on this analysis, 89 predicted viral contigs were found, including two complete pro-viral genomes, four high-quality (>97% completeness) viral genomes, and 12 medium-quality (>50% completeness) viral genomes. A further 32 viral contigs of low quality (representing a fragment of a viral genome) were validated using the IMG viral database (Supplementary Table S3). Mapping the extracted viral contigs to the metagenomic data revealed that the bacterial communities in bulbs from Washington and Georgia had distinct viral communities in terms of composition and abundance. The Washington bulbs exhibited a greater abundance (absolute counts) and diversity (number of viral contigs present) of viral contigs (Fig. 6; Supplementary Fig. S3). Indeed, the symptomatic bulbs from Washington contained 71 of the 89 identified viral contigs, as opposed to 48 identified in the symptomatic onion bulbs from Georgia, with 30 low-abundance viral contigs shared between bulbs from the two states. None of the viral contigs was detected in the metagenomic data for the asymptomatic bulbs from either state, which is consistent with the hypothesis that the greater bacterial load in infected onion bulbs (as suggested by the amplicon and metagenomics sequencing results) is associated with a greater detectable viral yield.

Of the most abundant viral contigs in the Washington symptomatic bulbs, the top two mapped to unknown *Caudovirales* genomes from the IMG viral database, with *Rahnella* and *Pseudomonas* as predicted bacterial hosts, whereas the third most abundant viral contig was a complete proviral genome associated with *Pantoea*. These results are consistent with the presence of MAGs for these bacterial genera in the metagenomic data. By comparison, the most abundant viral contig in the data from symptomatic onion bulbs in Georgia mapped to an unknown Brunovirus, with *Burkholderia* as a host, which again was consistent with the MAG abundance and metagenomics results that showed *Burkholderia* to be the dominant pathogen in bacterial communities detected in the symptomatic bulbs from Georgia. To assess the potential host range of the viral contigs identified from the metagenomic data, the viral contigs were mapped against the vContact2 database to find known viruses with common marker genes, and the hosts from which these viruses have been identified using the Viral-Host database (https://www.genome.jp/virushostdb/). The resulting network of connections (Fig. 7) showed that 55 viral contigs could be linked to viruses with known bacterial hosts, most of which have been associated previously with foodborne bacterial pathogens of humans and plant-pathogenic bacteria. This would include, for example, species of *Escherichia* (Ngene et al. 2020) and *Salmonella* (Bumunang et al. 2023) as foodborne pathogens and species of *Enterobacter* (Schroeder et al. 2010) and *Burkholderia* (Gitaitis and Nischwitz 2007) as plant-pathogenic bacteria. Based on connections in the network, the viral contigs abundant in the symptomatic Georgia bulbs were inferred to have a much narrower

range of bacterial hosts compared with the abundant viral contigs from the symptomatic bulbs from Washington, as suggested by the greater number of connections between the latter and viruses with a diverse range of bacterial hosts. This difference also suggested that when screening the viral contig dataset against the IMG viral database, viral contigs in the Washington State metagenome mapped back to virus genomes with a more diverse range of hosts.

Together, these results suggest that viral populations associated with bacterial communities in symptomatic onion bulbs might be predating on the pathogenic bacteria in the communities, affecting disease progression. The difference in viral populations between symptomatic bulbs from Georgia versus Washington may reflect the distinct bacterial communities identified from symptomatic bulbs from the two states, although geographic region (state) was also confounded with differences in cultivar, farm, production practices, environmental conditions, and storage conditions. The absence of bacteriophages in the asymptomatic bulb samples suggests that the viral populations may be associated with the great abundance of pathogenic bacteria in the symptomatic bulbs and, therefore, actively predating on these bacterial hosts. Future work could focus on empirical validation of these interactions between bacteriophages and bacterial communities in onion bulbs by extracting the viral fraction at different stages of bulb development and bacterial rot progression to assess whether the viral fraction of the bulb bacterial communities plays a role in community dynamics and development of bulb rot. Analysis of possible AMGs present in the predicted viral contigs using the DRAM pipeline detected 23 possible AMGs in seven viral contigs (Supplementary Table S4). Of the 23 putative AMGs, 21 were detected in prophages, including Scaffold_24, which contained the highest number of putative AMGs (nine genes), followed by Scaffold_13 (four genes) and Scaffold_97 (four genes). This result suggests that the prophages identified in this study might have historically played a role in improving the fitness of the bacterial host in which they were integrated (Wendling et al. 2021). This hypothesis is further suggested by the predicted functions of the putative AMGs, which included genes involved in energy acquisition, carbon utilization, and nutrient transport (Supplementary Table S4). In particular, Scaffold_24 contained three putative ABC transporter genes for sulfonates, which have been previously documented as important for biofilm formation and virulence of the citrus pathogen *Xanthomonas citri* pv. *citri* (Pereira et al. 2015). It is also important to note that Scaffold_24 was predicted to target a *Pseudomonas* host, suggesting that this prophage might be important for the fitness of the *Pseudomonas* to infect the onion core. Indeed, several studies have previously highlighted the crucial role that prophages play in the fitness and virulence of *Pseudomonas aeruginosa* through the use of AMGs, including genes involved in toxin production and biofilm formation (James et al. 2012; Rice et al. 2009; Tsao et al. 2018).

Conclusions. In this study, we aimed to characterize the diversity and functional potential of bacterial communities associated with asymptomatic onion bulbs versus onion bulbs with bacterial rot symptoms that were harvested from a farm in each of two states, Georgia and Washington. The results suggest that bacterial bulb rot may not be driven by a single bacterial pathogen but may instead be influenced by cohabitation and interaction among several bacteria. The presence of bacteria in *Acetobacterales* in many of the symptomatic onion bulbs further hints at a temporal dimension to progression of bacterial bulb rot that was beyond the scope of this study. Considering the lack of onion pathogenicity genes found in the *Acetobacter* MAGs compared with the other genomes extracted from the metagenomics data, one can hypothesize that the initial stages of infection by traditional bacterial pathogens (e.g., *Pantoea*,

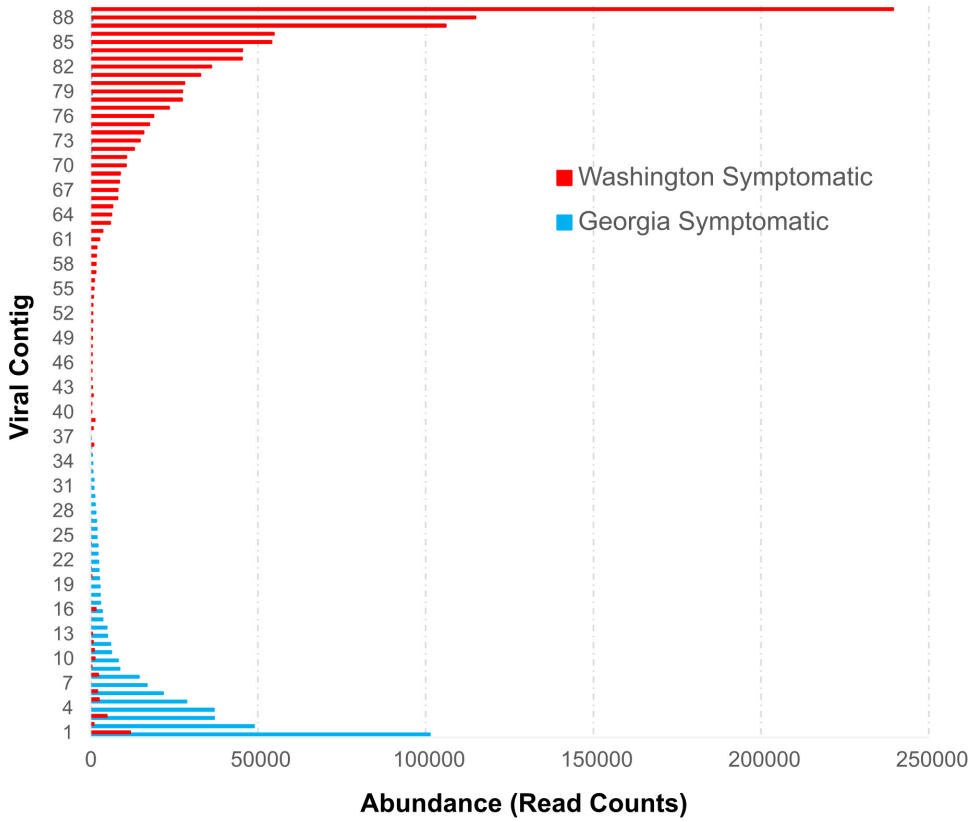

Fig. 6. Abundance (expressed as absolute counts) of 89 viral contigs identified in the metagenome data from symptomatic onion bulbs (bacterial rot) in Georgia and Washington State.

Burkholderia) may result in disruption of onion bulb tissue and defenses, so that opportunistic bacteria can then establish in the disrupted tissue. This study also showed that the geographic location of onion crops, in this case Georgia and Washington, could have a significant impact on the primary bacterial pathogenic communities causing bacterial bulb rot, although geographic location in this study was confounded with many other factors, including cultivar, environmental conditions in a winter versus summer growing season, onion production practices, and bulb storage conditions. Bacterial pathogens that cause onion bulb rot during storage primarily are introduced to the necks and bulbs toward the end of the field season (Dutta et al. 2016; Schwartz and Mohan 2008), so the dominant bacterial communities identified in the symptomatic bulbs from the two states may be a major driving force affecting disease progression. Future studies could, therefore, correlate the bacterial communities present in symptomatic onion bulbs with the prevalence of these microbiota in aboveground onion plant tissues to better inform potential management practices. Furthermore, the bacterial function examined in this study highlighted potential mechanisms of survival of the dominant pathogenic bacteria to common practices used in onion production, such as the application of copper bactericides, and, therefore, could be valuable for the development of preventive strategies to reduce losses to bacterial bulb rot. The viral populations detected in the symptomatic onion bulbs in this study could potentially be used as biocontrol agents. Future studies should assess the role of these viral populations in progression and prevention of bacterial bulb rot of onion.

Limitations of the study. This study was hampered by the overwhelming host-related genetic signals in the DNA data, which limited the ability to capture and describe the total biodiversity of the bacterial communities associated with onion bulbs. In addition, the study was limited to a small set of bulbs sampled at a single time from a single cultivar stored in a single storage facility, for each of two states. Despite these limitations, this study represents the most detailed characterization of endophytic bacterial communities associated with onion bulbs to date and serves as a framework for future studies to address more detailed aspects of the progression of bacterial bulb rot of onions.

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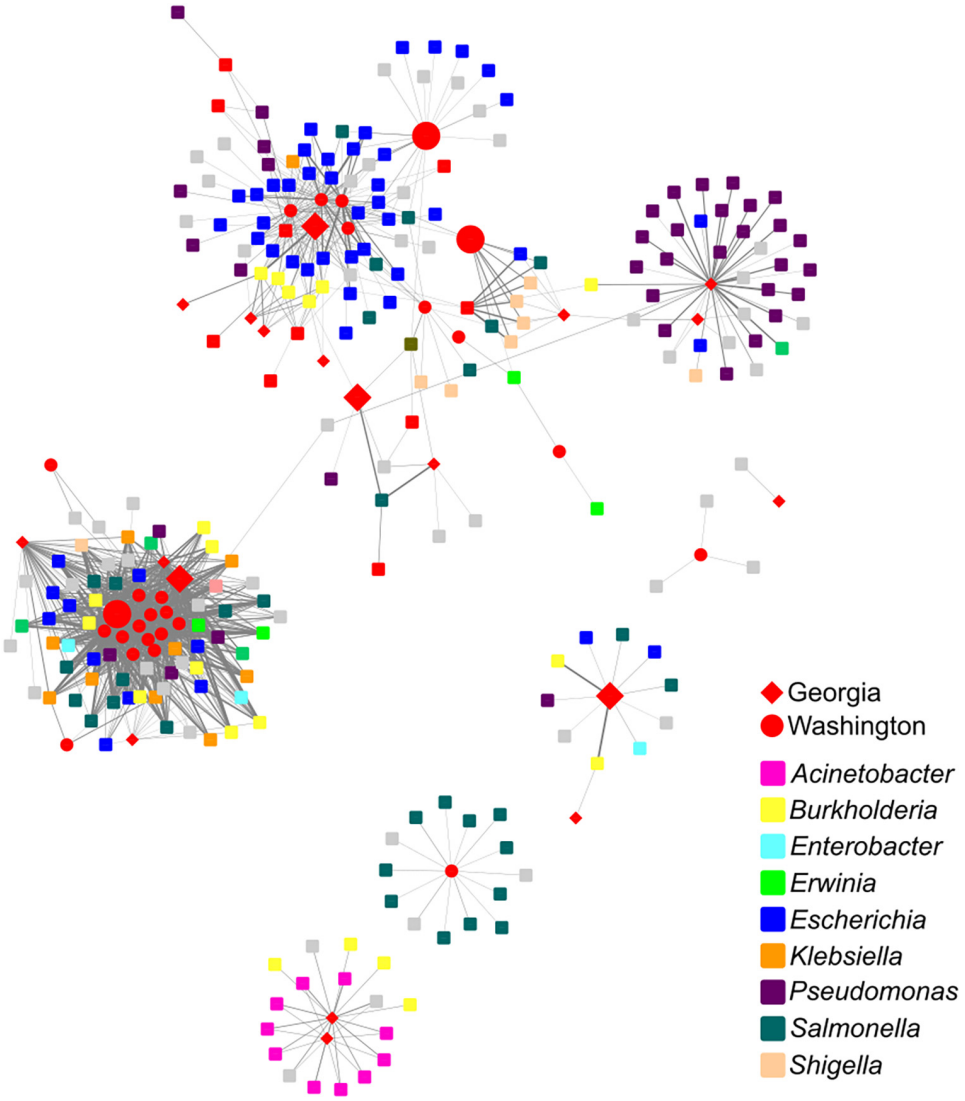

Fig. 7. Network of connections between the viral contig data and known viral genomes from the vContact2 database, based on shared viral genes. Viral contigs from the data in this study are red and shaped as diamonds or circles, according to the metagenomic data for bulbs produced in Georgia and Washington State, respectively. Nodes for the viral genomes from the vContact2 database are colored according to the host with which these viruses are associated. The width of the connections between nodes (edges) is a function of the strength of the correlation between the viral contigs and the viral genomes in the database, with thicker connections representing stronger links between the nodes.

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